Original article

On the importance of adequately choosing the ingredients of yoghurt and enriched milk for their antioxidant activity

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Summary The antioxidant activity of several dairy products, yoghurt enriched with green tea and lemon, fermented milk, yoghurt with strawberry pulp, 'low-calorie' yoghurt with inulin and milk enriched with vitamin E and their ingredients were analysed. Yoghurt enriched with green tea and lemon showed the best lipidic antioxidant capacity. All the dairy products analysed were very good OH· radical scavengers. The dairy products analysed were unable to scavenge H_2O_2 except green tea. The antioxidant activity of these samples resisted high temperatures in the Rancimat test; of the ingredients analysed, the best antioxidant activity was found for vitamin E followed by green tea, pectin, *Lactobacillus acidophilus*, lemon pulp and cornstarch. Antioxidant activity did not suffer variations during storage at an unfavourable temperature (40 °C), as demonstrated by the linoleic acid assay. Yoghurt enriched with green tea and lemon, yoghurt with strawberry pulp and low-calorie yoghurt with inulin produced the best results in the Trolox equivalent antioxidant capacity (TEAC) assay.

Keywords Antioxidants, cornstarch and vitamin E, enriched milk, free radicals, green tea, *Lactobacillus acidophilus*, lactic ferments, lemon pulp, pectin, skim milk, sweetener, yoghurt.

Introduction

Recent years have seen a gradual decline in milk drinking in its traditional form and an increase in the consumption of yoghurts, fermented milk products, and functional foods with a dairy base [http://www.mapya.es/es/estadistica/infoestad.htm; Ministerio de Agricultura, Pesca y Alimentación (M.A.P.A.) (2006)]. Many of the components of milk may be beneficial because of their antioxidant potential; for example, the enzymes dismutase superoxide and catalase (Lindmark-Mansson & Akesson, 2000). Casein seems to favour iron autoxidation by inhibiting lipid peroxidation (Cervato *et al*., 1999). Milk may also scavenge the superoxide anion, block the radical DPPH (1,1-diphenyl-2-picrylhydracyl) and OH· radicals (Suetsuna *et al*., 2000; Chen *et al*., 2003), and inhibit enzymatic and non-enzymatic lipid peroxidation probably due to the capture of free radicals (Rival *et al*., 2001; Chen *et al*., 2003). Casein contains a high quantity of potential antioxidant amino acids, such as tyrosine, tryptophan, histidine, lysine and methionine (Chen *et al*., 2003). The proteins found in milk whey, such as α-lactalbumin, β-lactoglobulin and lactoferrin, have also demonstrated antioxidant activity (Ostdal *et al*., 1996; Donnelly *et al*., 1998; O'Connell & Fox, 2001; Knowles & Gill, 2004). The possible antioxidant mechanisms of whey proteins include the scavenging of transition metals by lactoferrin and whey albumin and the scavenging of free radicals by amino acids, such as tyrosine and cysteine (Ostdal *et al*., 1996; Pihlanto, 2006). The antioxidant activity of whey proteins depends on the availability of the sulfhydryl groups, and it falls when these groups are blocked (Tong *et al*., 2000). Conjugated linoleic acid (CLA) also acts as antioxidant, even more so than ascorbic acid, α-tocopherol and buthylated hydroxytoluene (BHT) (Ha *et al*., 1990), an effect that may be responsible for its antiaterogenic activity (Pfeuffer & Schrezenmeir, 2000). Uric acid shows ferric ion-reductant activity (Chen *et al*., 2003) and is an effective antioxidant in preventing the light- and peroxidase-induced oxidation of milk, being similar in this respect to ascorbate (Ostdal *et al*., 2000). Vitamin C, vitamin E and carotenoids are recognised antioxidants in milk (Pulido *et al*., 2003).

The antioxidant activity of these lactic bacteria is demonstrated by their inhibition of ascorbate and linoleic acid autoxidation, their iron-scavenging and -reducing capacity, and the capture of reactive oxygen species, their Mn-dismutase superoxide activity and the fact that they contribute to reduced glutation, a powerful cell antioxidant (Lin & Yen, 1999; Kullisaar *et al*., 2003).

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In particular, in recent years a number of studies have focused on the capacity of tea, one of the most widely consumed drinks in the world, to elicit antioxidant protection in humans (Nardini *et al*., 2002). Furthermore, a number of fruits have been tested for their *in vitro* and *in vivo* antioxidant activity; strawberries and citrus fruits were found to be good sources of natural antioxidants. In addition to the usual nutrients, such as vitamins and minerals, strawberries are also rich in anthocyanins (a source of natural colourants) (Murcia *et al*., 2006). Of the other main prebiotics, inulins are nondigestible food ingredients that have beneficial effects on the growth and activity of bacteria in the colon. The main sources of inulin and oligofructose that are used in the food industry are chicory and artichoke (Roberfroid, 2000; Kaur & Gupta, 2002).

Consumers believe that foods rich in antioxidants may afford a degree of protection against free radical damage not only in foods, but also in the human body, where they offer protection against cardiovascular diseases, damage to nucleic acids and other deteriorative processes (Shi & Noguchi, 2003). Although data exist on the antioxidant activity of individual components of milk, tea, fruit, etc., it is necessary to investigate the total antioxidant capacity (TAC) without distinguishing the individual components and in different biological systems (Chen *et al*., 2003). In this respect, there is little information on the effect produced by adding tea extract, fruits or oligofructose as ingredient to fermented milk. These novel dairy products illustrate quite well the tendency of the market, with the incorporation of ingredients known to have antioxidant activity. The aim of this incorporation is to increase the functionality and antioxidant activity of these foodstuffs and in this way to improve the consumer's protection against pathologies related with free radicals.

The first aim of the present study was to evaluate the antioxidant activity and free radical scavenging of novel dairy products (yoghurt enriched with green tea and lemon, fermented milk, yoghurt with strawberry pulp, 'low-calorie' yoghurt with inulin and milk enriched with vitamin E); the second was to evaluate the contribution to antioxidant activity of the different added ingredients and the maintenance of antioxidant activity during product shelf-life and even at 120 °C to mimic what would happen when consumers use these products to elaborate other foods such as sauces or cakes.

Materials and methods

Samples

Five dairy products with a quite good acceptance by the consumer and available in the market were analysed: (i) yoghurt enriched with green tea and lemon; (ii) fermented milk; (iii) yoghurt with strawberry pulp; (iv) 'low-calorie' yoghurt with inulin; and (v) milk enriched with vitamin E. The ingredients used to manufacture the dairy products by the industry (skim milk, lactic ferments: *Lactobacillus bulgaricus, St. thermophilus*; *Lactobacillus acidophilus*, pectin, lemon pulp, green tea, sweetener and cornstarch) were obtained from a dairy product manufacturer (Corporación Alimentaria Peñasanta, Asturias, Spain).

Standards used as control were propyl gallate (E-310), buthylated hydroxyanesole (BHA; E-320), buthylated hydroxytoluene (BHT; E-321) and the chemicals used were of analytical grade available and were purchased from Sigma Chemical Co. (Poole, Dorset, UK).

The dairy products and the milk enriched with vitamin E were added directly in every assay using the amounts specified for every method, except in the Rancimat test when 50% concentrations were used. The ingredients were prepared at the concentrations at which they are added to elaborate the dairy products. The widely used food antioxidants, BHA (E-320), BHT (E-321) and propyl gallate (E-310), were used at the permitted commercial concentration of 100 $\mu g\ g^{-1}$ (FAO/WHO, 1999).

Methods

Peroxidation of phospholipid liposomes

The ability of compounds to inhibit lipid peroxidation at pH 7.4 was tested using ox brain phospholipid liposomes, as described in Martínez-Tomé *et al*. (2004). The experiments were conducted using a physiological saline buffer (3.4 mM Na_2HPO_4–NaH_2PO_4, 0.15 M NaCl, pH 7.4). In a final volume of 1 mL, the assay mixtures were made up with phosphate-buffered saline (PBS), 0.5 mg mL^{-1} phospholipid liposomes, 100 μM $FeCl_3$ and 100 μL of the tested samples (or 100 μL of food common antioxidants dissolved in water), and 100 μM ascorbate (added last to start the reaction). Because BHT is not fully soluble in aqueous solution, and its emulsion is not homogeneous, deionized water with a conductivity of not more than 4 $\mu S\ cm^{-1}$ was used to dissolve it. Incubations were carried out at 37 °C for 60 min. At the end of this incubation period, 1 mL each of 1% (w/v) thiobarbituric acid (TBA) and 2.8% (w/v) trichloroacetic acid were added to each mixture. The solutions were heated in a water bath at 80 °C for 20 min to develop the malondialdehyde-thiobarbituric adduct [$(TBA)_2$-MDA]. The $(TBA)_2$-MDA chromogen was extracted into 2 mL of butan-1-ol and the extent of peroxidation was measured in the organic layer as absorbance at 532 nm.

Hydroxyl radical scavenging

In a final volume of 1.2 mL, the reaction mixtures contained the following reagents: 10 mM KH_2PO_4–

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KOH buffer (pH 7.4), 2.8 mM H_2O_2, 2.8 mM deoxyribose (where used), 50 μM $FeCl_3$ premixed with 100 μM ethylenediaminetetraacetic acid (EDTA) before addition to the reaction mixture, and 100 μL of the tested samples (milk enriched with vitamin E, dairy products and their ingredients) and additives dissolved in water. Ascorbate (100 μM), where used, was added to start the reaction. The tubes were incubated at 37 °C for 1 h. The products of the $OH^{\cdot}$ radical attack on deoxyribose were measured as described in Murcia *et al.* (2002).

Hydrogen peroxide scavenging

The samples to be tested with H_2O_2 dissolved in water were incubated with 0.84 mM H_2O_2 for 10 min at 25 °C. Aliquots of these compounds were then taken and assayed for remaining H_2O_2 by using the peroxidase system (Martínez-Tomé *et al.*, 2001). The remaining H_2O_2 was measured by the formation of a chromophor recorded at 436 nm in reaction mixtures containing, in a final volume of 1 mL, 0.15 M KH_2PO_4–KOH buffer (pH 7.4), 50 μL guaiacol solution (prepared by adding 100 μL of pure guaiacol liquid to 100 mL of water) and 10 μL of Sigma type IV horseradish peroxidase (Sigma, Poole, UK) (5 mg mL^{-1} in the same phosphate buffer). *N*-Acetyl-L-cysteine (NAC) was used as a positive control of hydrogen peroxide scavenging.

Rancimat test for oxidative stability

Sample preparation in the Rancimat test consisted of macerating the different samples and antioxidants with 25 g butter or olive oil for 3 h at room temperature before analysis. The milk and dairy products were used at 50% (w/w) concentration, while the widely used antioxidant additives, BHA, BHT and propyl gallate, were used at the permitted commercial concentration of 100 μg g^{-1} and the ingredients at the same concentrations as used in dairy products.

All oxidative stability measurements were performed with a Rancimat apparatus (Metrohm model 743, Herisan, Switzerland) by measuring the induction period of butter (or olive oil) with or without the addition of the tested compounds, using the automated swift test. Determination of the induction period, at 120 °C and with an air flow rate set of 20 L h^{-1}, was based on the detection of volatile acids. The induction period is considered as the time elapsed until reaching the inflection point of the conductivity vs. time curve recorded by the Rancimat (Murcia *et al.*, 2004).

The relative activity of the antioxidants is expressed by the protection factor (PF), oxidative stability or antioxidant index, which is calculated by dividing the induction period (IP) of butter or oil with added antioxidants, by the induction period of the control (butter or olive oil alone).

Determination of antioxidant activity in the linoleic acid system

To a solution of 10 mL of linoleic acid (11.7 g L^{-1} in 99.8% ethanol) and 10 mL of phosphate buffer (200 mM, pH 7.0), 5 mL of the analysed sample (or 5 mL of the common food antioxidants dissolved in water) was added. The total volume was adjusted to 25 mL with deionized water. This solution mixture was incubated at 40 °C, and the degree of oxidation was measured. For this, 10 mL of ethanol (75%), 0.2 mL of an aqueous solution of ammonium thiocyanate (30%), 0.2 mL of sample (solution mixture), and 0.2 mL of ferrous chloride solution (20 mM in 3.5% HCl) were stirred for 3 min. The absorption values of the mixtures measured at 500 nm were taken as the peroxide content. The inhibition percentage of linoleic acid peroxidation, 100 − [(Abs increase of sample/Abs increase of control) × 100] was calculated to express antioxidant activity (Murcia & Martínez Tomé, 2001).

Measurements of total antioxidant activity by TEAC assay

The 2,2′-azino-bis-(3-ethylbenzo-thyazoline-6-sulphonic acid) diammonium ($ABTS^{\cdot -}$) radical solution was generated from the following reagents: 2.5 mM 2,2′-azobis (2-amidinopropane) hydrochloride (ABAP) and 20mM $ABTS^{2-}$ stock solution in phosphate buffer solution (containing 100 mM phosphate and 150 mM NaCl, pH 7.4). These were incubated at 60 °C for 12 min, protected from light, and stored at room temperature. The absorbance at 734 nm was measured to check $ABTS^{\cdot -}$ formation (the results must be between 0.35 and 0.45) (Parras *et al.*, 2006). The antioxidant activity of the samples analysed (40 μL mixed with 1960 μL of the radical solution) was measured at 734 nm for 6 min. The decrease in absorption at 734 nm observed 6 min after the addition of each compound was used to calculate the Trolox equivalent antioxidant capacity (TEAC).

A calibration curve was prepared with different concentrations of Trolox (standard solution used to evaluate equivalent antioxidant capacity). By measuring the increase in absorption during 6 min (standard range of 0–10 μM), the absorbance values were corrected for the solvent [samples giving absorption < standard (at 10 μM) were diluted to reduce the measurement within the appropriate part of the Trolox standard curve].

$$\Delta Abs_{Trolox} = Abs_{t=6min\,Trolox} - Abs_{t=6min\,solvent}$$

The regression coefficient (rc) was calculated from the calibration curve.

$$\Delta Abs_{Trolox} = rc \times [Trolox]$$

To establish the TEAC of commercial antioxidants or analysed samples, the increase in absorption was

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measured in the same way. The TEAC was calculated as follows:

$$\mathrm{TEAC}_{\mathrm{sample}} = \frac{\Delta \mathrm{Abs}_{\mathrm{sample}}}{\mathrm{rc}}$$

The TEAC represents the concentration of a Trolox solution that has the same antioxidant capacity as the analysed sample.

Data analysis

Data were analysed using the Statistical Package for Social Sciences Windows 9.0. The analyses of variance (ANOVA) were carried out after quintuplicate experiments, calculating the significance level by using the LSD multiple range test.

Results

Antioxidant capacity expressed as scavenger of peroxyl radical (LOO·)

The lypoperoxyl assay examines whether a substance inhibits the peroxidation of artificial lipid systems, such as brain phospholipid liposomes incubated with $FeCl_3$ and ascorbic acid, by scavenging peroxyl radicals. Evaluation with thiobarbituric acid and trichloroacetic acid was measured at 532 nm. A decrease in the absorption spectrum after the sample is added indicates that the sample scavenges LOO· radical (Murcia *et al.*, 2002).

Table 1 shows the inhibition of peroxidation by dairy products and their ingredients. Yoghurt enriched with green tea and lemon showed the best lipidic antioxidant capacity, followed closely by milk enriched with vitamin E and BHA. However, yoghurt enriched with green tea and lemon showed significant differences ($P < 0.05$) with the rest of the dairy products analysed (fermented milk, yoghurt with strawberry pulp and low-calorie yoghurt with inulin) and common food additives (propyl gallate and BHT).

Taking into account the antioxidant activity obtained for the different products, their ingredients were analysed in the amounts added to elaborate dairy products. The results show that two ingredients (skim milk and green tea) contribute to the high antioxidant activity in yoghurt enriched with green tea and lemon (Table 1). The rest of the added ingredients showed low antioxidant activity and differed significantly ($P < 0.05$) with respect to the dairy products, being in decreasing order, lactic ferments, sweetener, lemon pulp, cornstarch, pectin, *L. acidophilus* and vitamin E. Although skim milk is a good peroxyl scavenger, the above ingredients probably produce a dilution effect on the antioxidant capacity of the final product. With regard to milk enriched with vitamin E, the high antioxidant activity may be due to the skim milk, which is a much better ($P < 0.05$) peroxyl scavenger than vitamin E (analysed at the concentrations to prepare this type of milk).

Table 1 Inhibition of peroxidation in the lipid system using ox-brain phospholipids by dairy products and their ingredients, compared with the activity of common food antioxidants[a]

Added to reaction mixture	% Inhibition
None (control)	–
Samples	
Yoghurt enriched with green tea and lemon	81.7 ± 0.1
Fermented milk	45.6 ± 0.1
Yoghurt with strawberry pulp	58.9 ± 0.1
Low-calorie yoghurt with inulin	41.2 ± 0.2
Milk enriched with vitamin E	77.9 ± 0.1
Ingredients	
Skim milk	65.3 ± 0.1
Lactic ferments	28.6 ± 0.2
L. acidophilus	12.2 ± 0.2
Pectin	20.0 ± 0.1
Lemon pulp	25.3 ± 0.1
Green tea	65.3 ± 0.1
Sweetener	25.9 ± 0.2
Cornstarch	22.2 ± 0.2
Vitamin E	4.4 ± 0.1
Standards	
BHA	71.4 ± 0.1
BHT	22.3 ± 0.1
Propyl gallate	57.4 ± 0.2

[a]Statistical differences were analysed by ANOVA ($P < 0.05$).

Antioxidant capacity expressed as scavenger of hydroxyl radical

Hydroxyl radicals are extremely reactive and may be generated under physiological conditions in the human body, where they react with non-selective compounds such as proteins, DNA, unsaturated fatty acids and almost every biological membrane. The deoxyribose assay is used to detect possible scavengers of OH· radicals, which are formed by a mixture of ascorbate and $FeCl_3$-EDTA. The products of the OH· attack upon deoxyribose were evaluated with thiobarbituric acid (Murcia *et al.*, 2001).

Table 2 shows the deoxyribose damage caused by OH· radical in the presence of dairy products. Yoghurt enriched with green tea and lemon, fermented milk, yoghurt with strawberry pulp, low-calorie yoghurt with inulin and milk enriched with vitamin E are very good OH· radical scavengers and do not show significant differences ($P < 0.05$) between them. In addition, these results are significantly better ($P < 0.05$) than those obtained with common food additives (BHA, BHT, propyl gallate).

The ingredients of the dairy products were also analysed at the amount used in dairy products (Table 2).

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Table 2 Deoxyribose damage caused by the OH· radical in the presence of dairy products and their ingredients compared with the activity of common food antioxidants[a]

	Damage to deoxyribose (A_{532nm})		
Added to reaction mixture	**RM+DR**	**% Inhibition**	**Omit ASC**
None (control)	1.24 ± 0.01	–	0.22 ± 0.01
Samples			
Yoghurt enriched with green tea and lemon	0.16 ± 0.02	87.0	0.04 ± 0.02
Fermented milk	0.19 ± 0.02	84.6	0.05 ± 0.02
Yoghurt with strawberry pulp	0.24 ± 0.01	80.8	0.10 ± 0.01
Low-calorie yoghurt with inulin	0.18 ± 0.03	84.7	0.05 ± 0.03
Milk enriched with vitamin E	0.15 ± 0.02	87.4	0.01 ± 0.02
Ingredients			
Skim milk	0.18 ± 0.01	84.8	0.04 ± 0.01
Lactic ferments	0.91 ± 0.05	22.5	0.14 ± 0.05
L. acidophilus	1.10 ± 0.04	11.3	0.13 ± 0.04
Pectin	0.98 ± 0.02	20.6	0.13 ± 0.02
Lemon pulp	0.85 ± 0.02	24.0	0.09 ± 0.02
Green tea	1.71 ± 0.01	–	1.43 ± 0.01
Sweetener	1.06 ± 0.03	13.6	0.12 ± 0.03
Cornstarch	0.85 ± 0.03	24.7	0.14 ± 0.03
Vitamin E	1.35 ± 0.04	–	0.23 ± 0.04
Standards			
BHA	0.90 ± 0.01	25.4	0.17 ± 0.01
BHT	1.12 ± 0.02	8.9	0.49 ± 0.02
Propyl gallate	1.42 ± 0.01	–	0.74 ± 0.01

[a]Statistical differences were analysed by ANOVA ($P < 0.05$).

The results show that skim milk is the only ingredient with a very high OH· scavenger capacity and it produced results similar to the dairy products studied. The protective effect obtained by the rest of the ingredients and common food additives were, in decreasing order, cornstarch = lemon pulp ≥ lactic ferments ≥ pectin > sweetener ≥ *L. acidophilus* (Table 2) all with percentages of around 20% inhibition. However, green tea and vitamin E were unable to scavenge OH· radicals and exhibited prooxidant activity similar to propyl gallate (Murcia *et al.*, 2002).

The third column in Table 2 shows that when ascorbate is omitted from the reaction, the level of OH· radicals generated is lower than when it is added; the absorption spectrum also decreases. The dairy products (yoghurt enriched with green tea and lemon, fermented milk, yoghurt with strawberry pulp, low-calorie yoghurt with inulin and milk enriched with vitamin E) scavenge OH·, lowering the absorbance even when ascorbate is omitted. They can therefore be considered as primary antioxidants. Similar results were also found for skim milk and the rest of the ingredients, which also produced a decrease in the absorbance, except in the case of green tea and vitamin E, for which the absorbance level was higher than in the control. It is probable that skim milk was mainly responsible for the above findings as it would contribute its high OH· radical scavenger activity to the final product.

As regards the common food additives analysed, according to Murcia *et al.* (2004), BHA acts as a primary antioxidant, while BHT exhibits secondary antioxidant activity because when ascorbate is omitted, the absorbance exceeds the control value. Its action mechanism may involve reacting with ascorbate, thus decreasing OH· generation. Propyl gallate is a prooxidant in the 'Omit ASC' column of Table 2.

Hydrogen peroxide scavenging

Hydrogen peroxide may be generated *in vivo* by several oxidase enzymes or by activated phagocytes during the killing of several bacterial and fungal strains. There is increasing evidence that H_2O_2, either directly or indirectly via its reduction product, OH·, may act as a messenger molecule in the synthesis and activation of several inflammatory mediators. H_2O_2 scavenging can be accurately measured by using the peroxidase-based assay, recording any decrease in the absorption spectrum after the compound is added to peroxidase–H_2O_2 mixtures (Martínez-Tomé *et al.*, 2001).

Table 3 shows the effect on H_2O_2 of dairy products compared with their ingredients. The results obtained for dairy products (yoghurt enriched with green tea and lemon, fermented milk, yoghurt with strawberry pulp, low-calorie yoghurt with inulin, milk enriched with vitamin E) demonstrate that they are unable to scavenge H_2O_2.

When individual ingredients were analysed, the only one to decrease the absorbance level was green tea, with 82.58% inhibition. This result is similar to NAC (used as positive control) and better than propyl gallate ($P < 0.05$), which is the only one of the common food additives analysed to show H_2O_2-scavenging capacity. The rest of the ingredients (skim milk, green tea, cornstarch, lemon pulp, lactic ferments, pectin, sweetener and *L. acidophilus*) did not scavenge H_2O_2, the respective absorbances exceeding that of the control.

These results obtained for the yoghurt and milk may be due to the different effects of heat treatment on several of the antioxidant enzymes that milk contains, such as superoxide dismutase (SOD), which catalyses the dismutation of superoxide anion to H_2O_2. This enzymatic activity is retained after pasteurisation. However, catalase which catalyses the decomposition of H_2O_2, is one of the most heat-labile enzymes occurring in milk. Glutathione peroxidase, which also removes H_2O_2, is not detected in market-pasteurised milk (Lindmark-Mansson & Akesson, 2000).

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Table 3 Scavenging of hydrogen peroxide by dairy products and their ingredients, compared with the activity of common food antioxidants, using peroxidase-based assays[a]

Added to reaction mixture	Absorbance (A_{436nm})
None (control)	0.44 ± 0.02
Samples	
Yoghurt enriched with green tea and lemon	0.61 ± 0.01
Fermented milk	0.52 ± 0.02
Yoghurt with strawberry pulp	0.50 ± 0.05
Low-calorie yoghurt with inulin	0.75 ± 0.03
Milk enriched with vitamin E	0.73 ± 0.02
Ingredients	
Skim milk	0.86 ± 0.04
Lactic ferments	0.61 ± 0.02
L. acidophilus	0.45 ± 0.03
Pectin	0.42 ± 0.01
Lemon pulp	0.43 ± 0.01
Green tea	0.07 ± 0.01
Sweetener	0.43 ± 0.03
Cornstarch	0.42 ± 0.04
Vitamin E	0.41 ± 0.02
Standards	
BHA	0.54 ± 0.01
BHT	0.49 ± 0.02
Propyl gallate	0.33 ± 0.02
NAC[b]	0.03 ± 0.03

[a]Statistical differences were analysed by ANOVA ($P < 0.05$).
[b]Used as positive control.

Table 4 Effect of dairy products and their ingredients, compared with the activity of common food antioxidants on the oxidative stability of butter expressed as protection factor (PF) tested by the Rancimat method[a]

Substance added to reaction mixtures	PF[b]
Samples	
Yoghurt enriched with green tea and lemon	1.06 ± 0.01
Fermented milk	1.09 ± 0.01
Yoghurt with strawberry pulp	1.06 ± 0.02
Low-calorie yoghurt with inulin	1.12 ± 0.02
Milk enriched with vitamin E	1.26 ± 0.01
Ingredients	
Skim milk	0.89 ± 0.01
Lactic ferments	0.88 ± 0.02
L. acidophilus	1.17 ± 0.01
Pectin	1.24 ± 0.01
Lemon pulp	1.17 ± 0.02
Green tea	1.34 ± 0.01
Sweetener	0.82 ± 0.01
Cornstarch	1.10 ± 0.02
Vitamin E	1.94 ± 0.01
Standards	
BHA	2.40 ± 0.02
BHT	1.40 ± 0.01
Propyl gallate	6.48 ± 0.01

[a]Rancimat tested at 120 °C. Statistical differences were analysed by ANOVA ($P < 0.05$).
[b]PF = IP (butter + samples)/(butter alone).

Rancimat results

The Rancimat test is used to obtain information on whether antioxidant activity resists heating at high temperatures. This assay is also applied to evaluate the protection that a sample provides to a food (rich in oils or fats) and to study whether manipulation or industrial processing with heat generates free radicals (Murcia *et al.*, 2001).

The PF expresses the oxidative stability of butter with dairy products and their ingredients (Table 4). The results, in decreasing order, were milk enriched with vitamin E > low-calorie yoghurt with inulin = fermented milk = yoghurt enriched with green tea and lemon = yoghurt with strawberry pulp which protect slightly increasing induction time. These results are lower ($P < 0.05$) than those obtained for common food additives (BHA, BHT, propyl gallate) analysed at the permitted concentration.

Of the ingredients analysed (at the amount used to elaborate dairy products), the those having the best antioxidant property was vitamin E, followed by green tea, pectin, *L. acidophilus*, lemon pulp and cornstarch. However, skim milk, lactic ferments and sweetener show prooxidant activity in this assay (Table 4), producing shorter induction times than control.

Although skim milk supports prooxidant activity in yoghurt enriched with green tea and lemon, other ingredients, like green tea and pectin, provide the final product with protection, increasing the stability of butter. On the other hand, in the milk enriched with vitamin E, which is elaborated with only these two ingredients, it is probably the vitamin E that provides good protection to the final product, and although skim milk does not protect it, the final antioxidant balance is positive and protects the stability of butter. This assay was also performed using olive oil (data not shown), the results not showing significant differences ($P < 0.05$) with respect to those obtained for butter.

Total antioxidant activity evaluation during storage

The method used to determine antioxidant activity during storage at unfavourable temperatures (40 °C) measures the inhibition of linoleic acid autoxidation during 28 days of storage. The inhibition of linoleic acid oxidation is measured at 500 nm with ethanol, ammonium thiocyanate and ferrous chloride. The inhibition percentage of linoleic acid peroxidation was calculated to express antioxidant activity: 100 − [(Abs increase of sample/Abs increase of control) × 100] (Murcia *et al.*, 2002).

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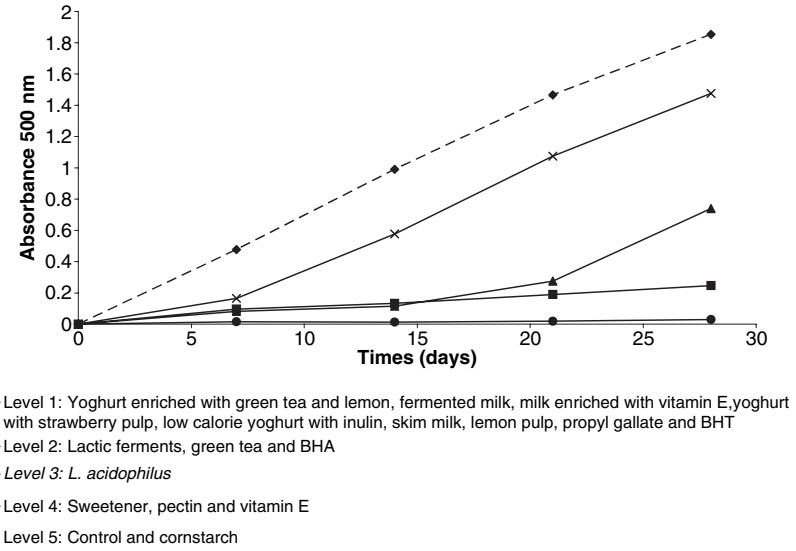

Figure 1 Absorbance at 500 nm for the oxidation of linoleic acid in the presence of dairy products and their ingredients and common food antioxidant during 28 days of storage.

Figure 1 shows the evaluation of the absorbance at 500 nm for the oxidation of linoleic acid in the presence of dairy products and their ingredients. The results are classified into five levels. The first level is attributed to very high antioxidant activity and is occupied by dairy products (yoghurt enriched with green tea and lemon, fermented milk, milk enriched with vitamin E, yoghurt with strawberry pulp and low-calorie yoghurt with inulin), the common food additives such as propyl gallate and BHT, and two of the ingredients, skim milk and lemon pulp. The high antioxidant activity of dairy products could be due to their common ingredient, skim milk.

Lactic ferments, green tea and BHA are included in the second level of antioxidant activity (Fig. 1). *L. acidophilus* is classified in the third level because, although this ingredient showed very high antioxidant activity until day 21, it decreased thereafter. The fourth level is assigned to products with a low antioxidant activity: sweetener, pectin and vitamin E. Finally, the fifth level is occupied by cornstarch, with absorbance values similar to the control sample. This ingredient is the only one that does not show a protecting activity in this assay, although this negative effect is not evident in the final product where it is used (yoghurt enriched with green tea and lemon and fermented milk) because these products show activity.

Total antioxidant activity evaluation

Dairy products are high in nutritional ingredients such as vitamins and phenolic compounds and flavonoids, which may produce additive or synergistic effects. The $ABTS^{\cdot-}$ radical solution was generated from ABAP and $ABTS^{2-}$ at 60 °C and the absorbance was measured at 734 nm. Subsequently samples were added and the decrease in absorption was measured after 6 min and 24 h. A TEAC (TEAC) value can be assigned to all compounds capable of scavenging the $ABTS^{\cdot-}$ by comparing their scavenging capacity with that of Trolox (vitamin E analogue, water-soluble). Quantitative evaluation of the antioxidant capacity using TEAC can be used to provide a ranking order of antioxidants. A calibration curve was prepared with different concentrations of Trolox to calculate TEAC (Murcia *et al.*, 2002).

Table 5 shows the TEAC values obtained for dairy products and their ingredients, by measuring the $ABTS^{\cdot-}$ scavenging capacity. In decreasing order, yoghurt enriched with green tea and lemon, yoghurt with strawberry pulp and low-calorie yoghurt with inulin produced the best TEAC results after 6 min, differing significantly ($P < 0.05$) from the other dairy products, such as fermented milk and milk enriched with vitamin E. However, when the values were measured after 24 h, all the values, even the lowest, increased and the products could be considered as very good $ABTS^{\cdot-}$ scavengers. According to Murcia *et al.* (2004), slow- and fast-acting antioxidants exist. TEAC is partially dependent on the number of free phenolic hydroxyls and is also affected by the type of linkage structures in the food matrix.

As regards the ingredients, which were analysed in the amounts to elaborate the dairy products, the highest value was recorded for green tea at 6 min time. The value was similar to that of propyl gallate, although in green tea the value slightly decreased after 24 h. This ingredient probably provides antioxidant activity to the yoghurt enriched with green tea and lemon. Skim milk and *L. acidophilus* produce TEAC values (6 min) of

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Table 5 Scavenging of ABTS radical anions by dairy products and their ingredients compared with the activity of common food antioxidants[a]

Substance added to reaction mixtures	TEAC[b]	TEAC[c]
Samples		
Yoghurt enriched with green tea and lemon	11.39 ± 0.01	14.55 ± 0.01
Fermented milk	1.32 ± 0.02	10.96 ± 0.01
Yoghurt with strawberry pulp	10.18 ± 0.02	15.86 ± 0.02
Low-calorie yoghurt with inulin	8.55 ± 0.02	13.27 ± 0.01
Milk enriched with vitamin E	1.92 ± 0.02	12.67 ± 0.02
Ingredients		
Skim milk	2.53 ± 0.01	6.76 ± 0.02
Lactic ferments	0.14 ± 0.02	1.63 ± 0.02
L. acidophilus	2.76 ± 0.02	5.30 ± 0.02
Pectin	0.06 ± 0.01	0.63 ± 0.01
Lemon pulp	0.40 ± 0.01	1.99 ± 0.01
Green tea	17.32 ± 0.01	15.72 ± 0.01
Sweetener	0.29 ± 0.02	1.22 ± 0.02
Cornstarch	–	–
Vitamin E	0.40 ± 0.01	0.77 ± 0.01
Standards		
BHA	0.44 ± 0.01	1.41 ± 0.02
BHT	0.26 ± 0.01	0.72 ± 0.01
Propyl gallate	17.20 ± 0.02	17.44 ± 0.02

[a]Statistical differences were analysed by ANOVA ($P < 0.05$).
[b]TEAC is the micromolar concentration of a Trolox solution showing the antioxidant capacity equivalent to the dilution of the substance under investigation at 6 min.
[c]TEAC is the micromolar concentration of a Trolox solution showing the antioxidant capacity equivalent to the dilution of the substance under investigation at 24 h.
–, Not detected.

around 3, although at 24 h the values reach around 6. The rest of the ingredients (lactic ferments, pectin, lemon pulp, sweetener and vitamin E) showed low $ABTS^{\cdot-}$-scavenging activity (6 min and 24 h) with values similar to BHA and BHT. Only cornstarch showed no TEAC.

Discussion

Few investigations have been carried out into whole milk and milk products. Those that have been carried out usually analyse individual components, e.g. milk, skim milk, whey, casein and lactoferrin, observing that they inhibit lipid peroxidation and peroxyl/superoxide radical generation, similar to our findings (Taylor & Richardson, 1980; Korpela *et al*., 1995).

In this context, it is important to note that nutrients are not consumed in isolation, and there may be physiological interactions and combined effects (Pfeuffer & Schrezenmeir, 2000). Milk should be considered in the hydrophilic plus lipophilic phases to analyse the contribution of other molecules to antioxidant activity. Added extracts with vitamin E and carotenoids could contribute to the antioxidant activity of the lipophilic phase, whereas vitamin C contributes such activity to the hydrophilic phase (Pulido *et al*., 2003).

Our TEAC results agree with the antioxidant capacity detected in bovine milk and whey (ABTS method) by Chen *et al*. (2003), who identified casein as a major $ABTS^{\cdot+}$ scavenger in milk. They also demonstrated that this activity apparently increased with increasing pH. Some of the ferric-reducing components of whey were identified, such as urate. Furthermore, heat treatment increased the total antioxidant capacity (ABTS) of whey, because the denaturation of proteins exposed initially buried reactive sites. Protein fractions and protein-free whey were found to be quite stable to heat treatment with respect to their ability to inhibit lipid peroxidation. However, Taylor & Richardson (1980) found that heat treatment seemed to decrease the antioxidant capacity of casein and Calligaris *et al*. (2004) observed the same for the reducing properties of milk, which were also time-dependent. However, only severe heat treatment, associated with the formation of brown melanoidins, permits the recovery and even a possible increase in milk antioxidant properties.

Casein also exhibits inhibitory action against Fe-induced peroxidation, probably favouring the autoxidation of iron and thus inhibiting lipid peroxidation. Phosphate confers $OH^{\cdot}$-scavenging activity on different casein molecules of α- and β-casein, while κ-casein produces less activity (Cervato *et al*., 1999). Our results obtained with the deoxyribose assay were very high for all the analysed samples which contained skim milk.

Caseins have a polar domain that contains phosphorylated serine residues -SerP-SerP-Glu-Glu, which effectively form complexes with calcium, zinc and iron (Díaz *et al*., 2003), accelerating the oxidation of ferrous iron by binding tightly to ferric ion (Galleher *et al*., 2005).

Lactoferrin also has a strong antioxidant effect in chelating metal (Nielsen *et al*., 2004), depending on the oxidation time. Lactoferrin and transferrin inhibited lipid peroxidation in a concentration-dependent manner. Lactoferrin also decreased the hydroxyl radical generation of phagocytes (Huang *et al*., 1999). Whey protein has also been shown to inactivate free radicals by their sulfhydryl group activity. Sufficient heat treatment of whey proteins leads to the formation of 'reactive' sulfhydryl groups that act as free radical scavengers, and thus as antioxidants (Galleher *et al*., 2005).

Wong & Kitts (2003) observed that buttermilk solids retarded the severity of lipid oxidation during propagation and produced a protective effect towards the $OH^{\cdot}$-induced oxidation of deoxyribose, because of the $OH^{\cdot}$ and peroxyl-scavenging activity of sulfhydryl groups and the sequestering of both Fe^{2+} and Fe^{3+}.

Conjugated linoleic acid, analysed *in vitro* upon long-term (15 days) incubation at 40 °C, was less prone to

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peroxide formation than linoleic acid (Pfeuffer & Schrezenmeir, 2000, 2006). Lin & Yen (1999) demonstrated antioxidative activity in lactic acid bacteria (*L. acidophilus*, *L. bulgaricus*, *S. thermophilus*, *Bitidobacterium longum*) which scavenge reactive oxygen species ($OH\cdot$, H_2O_2) and show reducing activity. Organisms capable of producing catalase or peroxidase can degrade H_2O_2. The chelating ability (Cu or Fe) of lactic acid bacteria could be due to the physiological chelators. Furthermore, *S. thermophilus* and *L. delbrueckii bulgaricus* produced an antioxidative effect on the inhibition of linoleic acid peroxidation (Lin & Yen, 1999), and '*in vivo*' the inhibition of the low density lipoprotein oxidation (Terahara *et al.*, 2001). The importance of α-tocopherol in increasing the resistance of milk to oxidation has been shown by other researchers using two different approaches: one the supplementation of cow feed and the other adding α-tocopherol directly to the cow milk (Lindmark-Mansson & Akesson, 2000).

In conclusion, it is important to emphasize the antioxidant activity of yoghurts incorporating new ingredients and fermented milks, as well as the correct selection of ingredients to increase their antioxidant functionality. It is clear that the consumer will obtain great benefits from these products in the form of increased protection against pathologies related with free radicals.

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